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POTENTIALS OF THE ISOLATED
FROG BRAIN

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY
AUGUST, 1941

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ELBERT TOKAJI

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INTRODUCTION

During the past two decades acetylcholine has become a substance of great significance in any discussion of nervous transmission. Much evidence now attests its indispensable function in the autonomic nervous system and at the myoneural junction. In recent years a lesser body of evidence indicates the possibility of its subserving important functions within the central nervous system.

Tetanzation of the isolated rabbit spinal cord releases an acetylcholine-like substance (Minz, 1936). Stimulation of the central vagus in the isolated dog head (Chang et al., 1937) or in the isolated central nervous system of the toad (Li, 1938) liberates acetylcholine from the central vagal endings; however, in intact animals Feldberg and Schriever (1936) and Adam et al. (1938) report negative findings upon stimulation of the vagus and other nerves. The latter did find an approximate doubling of the acetylcholine content of the cerebrospinal fluid upon stimulation of the hypothalamus, in seven of fourteen experiments.

Intracisternal application of acetylcholine causes a rise in blood pressure and augmentation of respiration in the dog (Suh, Wang and Lim, 1936); in the cat, intraventricular injection of 0.1 - 1.0 gamma results in respiratory arrest and occasional cardiac irregularities (Dikshit, 1934). In man, intraventricular injection of 2.5 - 7.5 mg. of acetylcholine produces nausea, vomiting and intestinal peristalsis (Henderson and Wilson, 1936), results potentiated by eserine and abolished by atropine; 10 - 50 gamma of eserine applied similarly produces like effects. Also

in man, intrathecal injection of 1 mg. of prostigmine causes loss of reflexes and tone (Kremer et al., 1937) while 1 mg. of eserine increases tone and reflexes and induces spontaneous pain; acetylcholine alone is ineffective but it is potentiated by subminimal doses of prostigmine; none of these actions is affected by atropine (Kremer, 1939).

Torda (1940) reports that both the contra- and homolateral extensor reflexes of the spinal toad are paralyzed by acetylcholine (0.01%) and prostigmine (0.25 mg.%). Bonnet and Bremer (1937a) find that acetylcholine (0.2 - 1.0 gamma) and eserine (50 - 200 gamma) augment the after-discharge of spinal reflexes in the toad and frog; larger doses of acetylcholine depress spinal centers. Acetylcholine (1:10,000) applied directly to the spinal cord of frogs or given intravenously in mammals (0.1 - 0.2 mg./kg.) depresses the excitability of intraspinal neurons in spinal frogs and mammals, increases it in thalamic frogs and mammals. Eserine (0.03 - 0.2 mg./kg.) markedly increases, acetylcholine (0.03 mg./kg. or over) and atropine (0.5 - 2.0 mg./kg.) generally depress the knee jerk in the cat (Schweitzer and Wright, 1937). Merlis and Lawson (1939), however, report that eserine (0.01 - 2.0 mg./kg.) is predominantly depressant to the knee jerk, but augmentory to the flexion and crossed extension reflexes in the dog.

As regards higher centers of the central nervous system, the evidence for acetylcholine action is far less conflicting. Acetylcholine (0.001 - 1.0 mg.) augments the response of the center for the linguo-maxillary reflex, as does eserine (Bonvallet and Minz, 1938b). One per cent eserine applied directly to the motor cortex of the cat elicits tremors and clonus of the contralateral limb and facilitates a succeeding faradization (Miller, 1937).

Electrical studies have shown that in the rabbit and cat, eserine (1%) increases the frequency of cortical potentials while diminishing the amplitude of preexistent slow waves and large fast ones as well, and that acetylcholine ($1:10^5$ - 1%) has a similar, though weaker, effect; acetylcholine (0.2 - 1%) applied to the eserinized cortex evokes diphasic spikes of large amplitude. These changes are associated with motor effects and both the spikes and motor effects are inhibited by previous atropinization (Miller et al., 1938 and 1940). Eserine (1%) increases both amplitude and frequency of small cerebellar waves and leads to the appearance of enlarged waves in groups or as scattered spikes; acetylcholine (1:2000 - 1:1000) enhances these effects and the accompanying changes of tonus; atropine abolishes them when present or prevents their occurring (Miller, 1941). Bonnet and Bremer (1937b) find that intracarotid injection of one-tenth of a gamma of acetylcholine increases the amplitude and frequency of spontaneous cortical potentials and reinforces the after-discharge of potentials evoked by sound in the acoustic cortex of the cat. Stimulation is followed by depression even with this amount, and larger doses are only depressant. Finally, Moruzzi (1939) has shown that intracarotid injection of two-tenths of a gamma of acetylcholine lowers the threshold of excitability of the masticatory cortex of the cat in the same manner as does stimulation of an associated facilitating center.

The evidence outlined above would suggest that a neurohumoral agent may be involved both in central synaptic transmission and in the inherent automatism of the central neurons. The present experiments on the isolated frog brain (which has already proven so useful in studying the control of the potential rhythm--Libet and Gerard, 1939; Gerard and Libet, 1940) were directed

toward further elucidation of these two problems by directly applying to the brain acetylcholine and drugs influencing its action, and studying the electrical consequences.

METHOD

Preparation, electrodes and recording. The brains of grass frogs (R. pipiens and R. foxinus) were isolated by decapitation, removal of overlying bone and section of nerves and dura mater and were then placed in Ringer solution in a small glass container. Ag-AgCl-wick electrodes led to push-pull, resistance-capacity coupled amplifiers and a crytograph which were used according to standard practise in this laboratory. One electrode was placed on the brain, the other at a distance from it in the solution. When recordings were made simultaneously from two regions, either a common indifferent electrode or two complete sets were used. Controls of the electrical system excluded the presence of common artifacts and proved the absence of interaction between the two active leads. (For preparation of the brain, electrodes, and composition of the Ringer solution see Libet and Gerard, 1939.)

Solutions. Acetylcholine chloride (Merck), eserine hydrochloride (Mallinckrodt), nicotine alkaloid (Elmer and Amend), atropine alkaloid (Merck) and cholinesterase (prepared from dried adrenal tissue by the method of Stedman et al., 1932) were all dissolved in Ringer solution and diluted to the desired concentration. Stock solutions of these drugs at appropriate concentrations remained potent and stable for several weeks without special precautions (1% acetylcholine was kept at a pH between 5.5 and 6.0). Solutions were adjusted to a pH of about 7.0 by the addition of HCl or NaOH before using, although controls proved that neither pH nor osmotic pressure was of significance within a wide range. The solution

surrounding the brain was always removed and replaced by a standard procedure, the old solution being sucked off and the electrodes and brain washed twice by flooding gently with 3 cc. of the new solution. Usually after 3 min. the solution was removed except for a shallow layer covering the lower one-fifth of the brain.

Temperature. Room temperature was at 22° - 23° C. for most experiments. When this rose above 26° C., it was found advisable to keep the frogs in ice water (about 10° C.) for half an hour before decapitation and to use cooled solutions for bathing the brain.

RESULTS

The effects of acetylcholine, eserine, nicotine, atropine and cholinesterase -- by themselves and in various combinations -- were explored on over one hundred brains.

Normal Rhythm

In the isolated brain the olfactory bulb generally has a 6 per sec. rhythm, with an extreme range of from 4 to 8. Its amplitude averages 0.03 to 0.1 mV., but may be exceptionally as high as 0.3 mV. The potentials of the cerebral hemispheres are much more variable than those of the bulbs in frequency, amplitude and regularity. Their frequency is mostly 6 to 8 per sec. and amplitude 0.02 - 0.03 mV., but with considerable moment to moment variation. In the great majority of cases electrical activity of the hemisphere ceases within an hour after isolation, even in the presence of stimulant drugs, while that of the bulb may continue for three hours or longer.

Reapplication of simple Ringer solution, in scattered instances, caused a distinct decrease in amplitude. This was particularly seen when the temperature was near 26° C. and with hemisphere rather than bulb potentials. Bulb potentials often increase

in both regularity and amplitude with time in Ringer solution, aside from the application of any test solution. These factors have been allowed for in evaluating the observations reported below. All observations refer to the olfactory bulbs unless otherwise noted.

Effects of Acetylcholine

The conflicting results in the literature, in some cases at least, might well be due to different concentrations of drugs used. A small difference in acetylcholine concentration, for example, is known to reverse its action. It seemed advisable, therefore, to explore all the drugs used over a wide range of concentration.

Acetylcholine as dilute as 10^{-8} has a pronounced effect. During 11 to 33 min., following a 3 min. bathing period, the amplitude of the bulb rhythm increases by 50 to over 300 per cent and is rarely maximal before 20 - 30 min. This is often accompanied by a decrease in frequency, from 6 to as low as 3 per sec., and by some enhancement of regularity. In 7 of 11 experiments, diphasic spike-like potentials of 0.1 to 1.0 mV. amplitude appeared in bulbs, hemispheres (mainly) or both. The long latency of the maximal response is not due to time for diffusion of the drug to a deep site of action, for bathing 10 min. rather than 3 did not alter this latency (2 experiments), nor did a tenfold increase in concentration (2 experiments). All these acetylcholine effects can be removed by washing with Ringer solution and then reproduced in the same brain by reapplication of the drug. 10^{-7} and 10^{-8} concentrations act alike.

Acetylcholine in 10^{-6} or 10^{-5} concentration is predominantly depressant (3 out of 4 cases), the amplitude of potentials fading gradually or suddenly and not being well restored by Ringer

solution. But in one case the action of 10^{-6} acetylcholine was similar to that of the weaker concentrations. A concentration of 10^{-4} acetylcholine enhances regularity and amplitude progressively over 20 - 30 min. without altering frequency, except for occasional large diphasic potentials. The effects can be removed by washing with Ringer solution and again produced with acetylcholine. At times, sudden depression may follow enhancement, an effect also removed after washing with Ringer solution. A similar action is observed with 10^{-3} acetylcholine, except that the depression after excitation is usually more pronounced.

TABLE 1

EFFECTS OF DIFFERENT ACETYLCHOLINE CONCENTRATIONS

Acetyl- choline Concen- trations	Bathing Time (min.)	Ampli- tude (per cent)	Pre- quency (per sec.)	Regu- larity	Diphasic Poten- tials (mV.)	Latency for Maximum (min.)
10^{-8}	10	100-150 increase	4-6	Moderately increased	None	30
10^{-8} or 10^{-7}	3	50-300 increase	3-6	Moderately increased	0.1-1.0	20-30
10^{-6} or 10^{-5}	3	25-100 decrease	6	Decreased	None	1-10
10^{-4} or 10^{-3}	1-3	25-100 increase	5-7	Greatly increased	0.1-0.2	15-35

Effects of Eserine

In concentrations of 10^{-8} through 10^{-5} eserine acts much as do similar concentrations of acetylcholine (exciting in the same concentrations as for acetylcholine, etc.); at higher concentrations its action differs markedly. A concentration of 10^{-4} produces a marked increase (100 to 300 per cent) in amplitude and

in regularity of potentials, maximal effects being achieved in 20-30 min. No depression follows excitation, although depression after excitation is seen with the same strength of acetylcholine. More concentrated eserine (10^{-3}) starts to lower frequency and increase amplitude and regularity of potentials, even after 1 min. bathing. This prompt effect progresses for as long as an hour after removal of the solution, during which time frequency may fall to as low as 1 per sec. and amplitude rise to as high as 1mV. Diphasic potentials frequently are seen in the middle and later phases of action. (See Table 2.)

Preceding eserinizatio~~n~~ modifies the effect of acetylcholine. (In the remainder of this section, wherever the word "acetylcholine" is used, a concentration of 10^{-8} is implied.) A concentration of 10^{-8} eserine reduces the time required for maximal acetylcholine action by 10 min. or more. Stronger eserine (10^{-6} or 10^{-5}) reverses the acetylcholine action so that it depresses rather than excites. (In one experiment this change did not occur and the acetylcholine acted exactly as if no eserine were present.) Eserine (10^{-8}) applied after acetylcholine may increase or decrease amplitude of potentials. (For these eserine effects refer to Table 3.)

Effects of Nicotine

Since nicotine blocks central synaptic transmission (Libet and Gerard, 1938; Schweitzer and Wright, 1938), this should abolish any acetylcholine action which depends on its functioning as a synaptic transmitter or as an adjuvant to transmission. Nicotine, in a concentration of 0.5 per cent, does abolish existing acetylcholine or eserine action (in concentrations of 10^{-8} through 10^{-3} for either drug) or prevent the inception of such action (see Table 3). Applied after acetylcholine or eserine, nicotine either

abolishes all potentials or leaves waves of reduced frequency (1 to 3 per sec.), greatly enhanced regularity, and either increased or decreased amplitude (dependent to some extent upon the time elapsed since decapitation and upon the amount of activity before application of nicotine). These altered waves rarely last for 10 min., but can often be restored by washing with Ringer solution or by readministration of acetylcholine or eserine. Such activity is never reminiscent of the previous acetylcholine or eserine potentials but resembles more closely those of nicotine alone.

TABLE 2
EFFECTS OF OTHER DRUGS

Drug	Amplitude (per cent)	Pre- quency (per sec.)	Regu- larity	Diphasic Potentials (mV.)	Time for Maximum (min.)
10^{-8} or 10^{-7} Eserine	100-300 increase	3-6	Moderately increased	0.1-0.5	20-30
10^{-6} or 10^{-5} Eserine	25-50 decrease	6	Decreased	None	1-10
10^{-4} Eserine	100-300 increase	5-6	Greatly increased	None	20-30
10^{-3} Eserine	100-1000 increase	1-4	Very greatly increased	0.1-1.0	10-45
0.5% Nicotine	50-300 increase	1-4	Very greatly increased	None	1-10
10^{-3} Nicotine	100-300 increase	4-6	Greatly increased	0.3-1.0	1-15
10^{-3} Atropine	100 decrease	0	Greatly decreased	None	1

By itself, 0.5 per cent nicotine greatly enhances the regularity of bulb potentials with a concomitant increase in amplitude. Frequency wanes with time (amplitude may also), but the waves continue regular to the end. Weaker nicotine stimulates. A strength of 10^{-3} (or less, at times) greatly enhances amplitude and regularity and sets off a long series of diphasic potentials of large amplitude (to 0.3 mV.) in bulbs and hemispheres. (Refer to Table 2.)

Effects of Atropine

The literature is conflicting concerning the influence of atropine on acetylcholine effects in the central nervous system. Frogs seem particularly insensitive to atropine, 500 mg./kg. being required to produce any general effect -- an enduring (7 - 48 hours) sensory and motor depression (Koppanyi, 1939). Bulb potentials, however, are increased 50 per cent in amplitude during the 10 - 20 min. following application of 10^{-5} atropine, while a concentration of 10^{-3} depresses them strongly (see Table 2).

In concentrations ranging from 10^{-3} to 10^{-5} , atropine abolishes the effects of 10^{-8} acetylcholine, alone or with 10^{-5} eserine; but 10^{-6} atropine enhanced the action of 10^{-8} acetylcholine in one case, depressed it in another. A combination of 10^{-5} atropine and 10^{-8} or 10^{-4} acetylcholine gradually depresses bulb potentials; washing with Ringer solution only partly restores them. (For these actions see Table 3.)

Effects of Cholinesterase

The greater concentration of cholinesterase in synaptic areas of the central nervous system, its increase in concentration coincident with the origin of synaptic function in embryos, and its ability to hydrolyze suitable amounts of acetylcholine within the brief refractory period of central neurons (Nachmansohn, 1940)

constitute evidence for a function of acetylcholine in central synaptic transmission. Whether as a physiological or pharmacological agent, it serves to test acetylcholine action.

TABLE 3
EFFECT OF OTHER DRUGS UPON ACETYLCHOLINE ACTION[§]

Drugs	Amplitude (per cent)	Frequency (per sec.)	Regularity	Diphasic Potentials (mV.)	Time for Maximum (min.)
10^{-8} Eserine, then 10^{-8} ACh ^a	100-200 increase	3-6	Moderately increased	0.1-0.3	10-20
10^{-5} Eserine, then 10^{-8} ACh	25-50 decrease	6	Little change	None	5-20
10^{-8} ACh, then 0.5% Nicotine	25-100 decrease	0-4	Greatly increased	None	1-2
10^{-3} ACh, then 0.5% Nicotine	100 decrease to 100 increase	0-3	Greatly increased	None	1-2
10^{-8} ACh + 10^{-5} Atropine	50-75 decrease	6	Little change	None	10-15
10^{-3} ACh + 10^{-5} Atropine	25-50 decrease	6	Little change	None	20-25
1:200 ChE ^a	50-100 decrease	6	Little change	None	15-30
10^{-3} ACh + 1:400 ChE	50-75 decrease	6	Little change	None	30-40
10^{-5} (10^{-3} ACh + 1:400 ChE)	50-75 decrease	6	Little change	None	30-35

[§] Compare with Tables 1 and 2.

^a ChE = cholinesterase, ACh = acetylcholine in the above Table.

The solution of cholinesterase used, dilution 1:200, is capable of hydrolyzing 2 mg. of acetylcholine in 25 - 30 min. and has no toxic or vasodilator action as tested by repeated injections in dogs (Schacter, 1941). In the dog, 1.5 cc. injected intravenously was able to abolish, over a period of 20 - 30 min., the cardioinhibition produced by mild stimulation of the peripheral vagus. While the preparation probably contains protein and,

perhaps, other impurities, the action on the frog brain may fairly be attributed to the enzyme.

In five experiments, this solution depressed bulb potentials progressively over 15 - 25 min., with no change in frequency or in regularity. Ringer solution or 10^{-8} acetylcholine largely reverses this action; 10^{-3} acetylcholine restores the brain potentials to normal, except for additional diphasic spikes (Table 3). In two experiments, the sequence of effects was successfully repeated on a single brain.

Cholinesterase (1:400), allowed to stand with 10^{-3} acetylcholine for half an hour before application to the brain, or the same mixture diluted 10^5 times further, gradually abolishes potentials in 35 - 40 min. Eserine (10^{-3}), even in the presence of cholinesterase (1:200), can partially restore the depressed potentials; or, applied to a fresh brain, gives a typical eserine action.

DISCUSSION

It is obvious that acetylcholine has a profound influence on the frog brain. Since minute amounts enhance spontaneous activity, as they do junctional transmission, and since added cholinesterase, which must decrease the "normal" concentration of acetylcholine, and eserine, which must increase it, respectively depress and enhance the potential waves, it seems probable that some synaptic transmission is involved in the maintenance of these rhythmic neuron potentials. This is further attested by the ability of nicotine, which blocks synaptic transmission, and of atropine, which specifically prevents the action of acetylcholine, to depress or abolish the spontaneous rhythm and to prevent the action of acetylcholine or eserine. The nicotine effect is all the more convincing since this drug does not abolish caffeine waves, shown

to be independent of synaptic transmission (Gerard and Libet, 1940), but does abolish the acetylcholine action which is presumably synaptic.

This work does not permit an adequate evaluation of the delay in action of the dilute drugs, especially acetylcholine, nor a quantitative estimate of the minimal effective concentrations. As acetylcholine diffuses into the brain substance from a surrounding solution, it is subject to continued destruction by the esterase present in the tissue; so that the final concentration reached at the points of action must be, in the extreme case, less than 10^{-8} above the normal level. And it may take some time to reach this level -- at least the more prompt action of acetylcholine on an eserinated brain, in which its destruction is lessened, suggests this. The in-diffusion of eserine would similarly raise the brain acetylcholine content, by an amount depending on the normal rate of acetylcholine liberation (Dikshit, 1938; Mann et al., 1938; Trethwie, 1938) and on the degree of inactivation of the esterase present.

Stronger concentrations of acetylcholine are well known to depress rather than stimulate synapses (Bonnet and Bremer, 1937b; Schweitzer and Wright, 1937), so the action of 10^{-6} and 10^{-5} concentrations of this drug need no further comment. Stronger eserine may likewise depress via a greater accumulation of acetylcholine. The action of still higher drug concentrations -- 10^{-4} or 10^{-3} acetylcholine or eserine, 0.5 per cent nicotine (see also the similar action of high Ca -- Dubner and Gerard, 1939; Libet and Gerard, 1939) -- in again augmenting potential amplitude and regularity and in slowing frequency is apparently independent of synaptic transmission and dependent on a change induced in the perikarya themselves. This is again suggestive of the action of strong

caffeine, and studies of pial-ventricular potentials under the influence of these high concentrations of the drugs in question will perhaps give an answer as to cell state.

In any event, acetylcholine in low concentration seems to enhance cell rhythms and synchrony by increased synaptic excitation (compare the action of moderate illumination of the eyes in increasing the synchrony of the optic rhythm in the cat --Dubner and Gerard, 1939). This is evidence, along with the ability of cholinesterase to abolish the spontaneous rhythm, that conducted impulses play a role in the normal synchronization of frog brain neurons. This mechanism is probably complementary to the electrical one demonstrated in the case of caffeine (Gerard and Libet, 1940) and perhaps involved in the action of the present drugs in high concentration.

SUMMARY

A study of the effects of acetylcholine, eserine, nicotine, atropine and cholinesterase on potentials of the isolated frog brain was directed toward further elucidation of mechanisms regulating brain potential rhythms.

Acetylcholine or eserine in weak concentrations (10^{-7} to 10^{-8}) augments synchronization of olfactory bulb potentials and often causes the appearance of large diphasic potentials. In concentrations of 10^{-5} or 10^{-6} either drug depresses or abolishes bulb potentials, while 10^{-3} or 10^{-4} acetylcholine or eserine (especially 10^{-3} eserine) markedly enhances their regularity and amplitude.

The latency of the acetylcholine response is appreciably reduced when the application of weak eserine solution precedes that of acetylcholine in similar concentration.

Both nicotine (0.5%), which blocks synaptic transmission, and atropine (10^{-5}), which specifically inhibits acetylcholine action, prevent the action of acetylcholine. Weaker nicotine stimulates; atropine in the concentration noted slightly increases the amplitude of olfactory bulb potentials.

Cholinesterase gradually decreases the amplitude of potentials, an effect which acetylcholine or Ringer solution reverses. When acetylcholine is mixed with adequate cholinesterase, only depressant action is observed.

The significance of these results as evidence of a synaptic mechanism for synchronization of brain potentials is discussed.

I wish to acknowledge at this time my great appreciation of the invaluable criticisms and suggestions of Dr. R.W. Gerard. I also wish to thank Mr. R. Schacter for preparing the cholinesterase used in these experiments.

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